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14. ABSTRACT
. Breast cancer refers to a variety of types that each derive from distinct causes and merit different treatments. It is crucial to develop new classes of molecules that target heretofore unexplored cellular mechanisms, so that tumors not responsive to current treatments might be responsive to new routes of therapy. We are exploring a previously under-appreciated avenue that can lead to precise identification of breast cancer sub-types to improve treatment options and identify new therapeutic targets. The genetic code is decoded by transfer RNA (tRNA). We discovered that breast tumors have high levels of tRNA and tRNA over-expression has an aberrant pattern suggesting that tumors use tRNA over-expression to mis-regulate the synthesis of some crucial proteins. Further, we found that a specific tRNA is an oncogene in breast cells suggesting that tRNA over-expression could even originate the development of breast cancer. We are identifying the mis-regulation targets of tRNA over-expression in breast cancer to serve as biomarkers and establishing the potential of targeting tRNA as a new route for breast cancer treatment.

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INTRODUCTION

Transfer RNAs (tRNAs) are small non-coding RNAs that read the genetic code in protein synthesis. They are essential for the proliferation, fitness, and adaptation of the cell. Previously, we showed that elevated tRNA levels are characteristic of breast cancer cells (1). Furthermore, over-expression of one specific tRNA - the initiator methionine tRNA - leads to increased cell proliferation and altered tRNA expression in a non-cancer breast epithelial cell line. Based on these results, we hypothesized that tRNA over-expression alters the translational regulation of key genes involved in cancer development and progression. We aim to (i) identify the protein or RNA targets that are mis-regulated upon tRNA over-expression, and (ii) determine the effect of tRNA over-expression on tumor initiation and progression.

BODY

This collaborative project involves equal efforts between the labs of both PIs. In general, the molecular work was performed in the Pan lab, and the cellular work was performed in the Rosner lab. This division of complementary efforts is outlined below according to the pervious and future work performed in either labs.

Task 1 – Identify gene targets whose translation is mis-regulated upon tRNA over-expression.
a) Establish the method: ribosome profiling. → **Completed successfully when the last annual report was written in September 2011.**
   Pan lab: performed the optimization of the ribosome profiling method.

b) Identify gene targets whose translation is mis-regulated upon tRNA over-expression. → **Near completion.**

To measure changes in translation upon tRNA over-expression, we initially planned to use stable cell lines over-expressing the initiator methionine tRNA ($tRNA_i^{Met}$) or the elongator methionine tRNA ($tRNA_e^{Met}$). Due to unexpected technical difficulties in maintaining these cell lines, we amended our experimental strategy to compare translation between non-tumorigenic breast cell lines and breast cancer cell lines. From our previous studies, we know that tRNA levels are highly elevated in the breast cancer relative to the non-cancer breast cell lines. We applied ribosome profiling to two non-cancer breast epithelial cell lines – 184 B5 and 184 A1 – and three breast cancer cell lines – MCF7, BT-474, and MDA-MB-231. Furthermore, we focused our analysis on differences in the ribosome density of 5'UTR regions since tRNA levels could significantly affect translational regulation for genes that contain upstream open reading frames (uORFs) in their 5' UTR (2).

We found that the ribosome density in the 5' UTR seems to vary significantly among the group of cell lines with similar tumorigenic properties (Fig. 1). The five lines we studied can be classified into three groups: 184A1 and 184B5 are non-tumorigenic (NT), BT474 is from a primary, non-metastatic tumor (TP), MDA-MB-231 and MCF7 are derived from metastatic tumors (TM). Comparing these three groups shows a surprising, unexpected result: the NT cells have similar ribosome density in 5'UTR among themselves, the TP cell has significantly lower ribosome density in 5'UTR than NT, but the TM cells have significantly higher ribosome density in 5'UTR than TP. Differential ribosome density in 5'UTR was also observed in the differentiation of mouse embryonic stem cells: compared to stem cells, differentiated cells have a significantly lower

ribosome density, suggesting a lower degree of translational regulation through 5' UTR after differentiation (3). Therefore, our result suggests that a primary tumor may also have decreased global regulation of translation when compared to non-tumorigenic or to metastatic breast cancer cells.
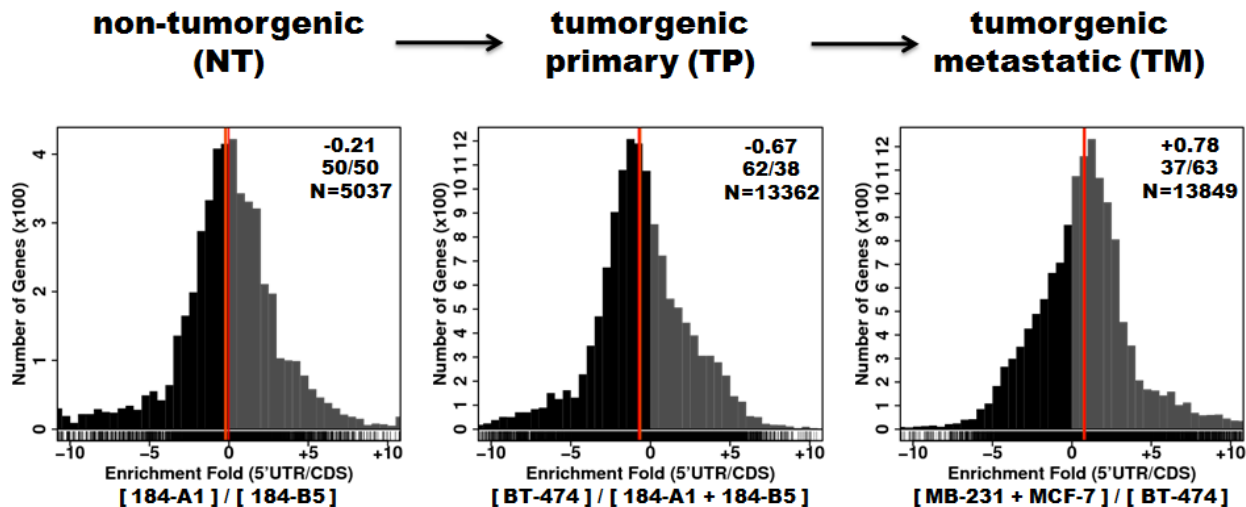
We next performed ribosome profiling of two cell lines derived from the same parent cell to directly address the idea of differential translation regulation as a function of tumorigenic status (Fig. 2). This test was necessary because the five lines we used above for ribosome profiling are derived from different people and therefore may have significant genetic differences. The line shCTGL-5 is highly metastatic, whereas shHMAG-6 is similar to a primary tumorigenic cell. We observed the same global difference as our previous result predicted, indicating that translational regulation has decreased significantly in primary breast tumors.

Fig. 1. *Comparing ribosome density in 5'UTR among three groups of breast and breast cancer cells.* TP cells have significantly lower 5'UTR density than NT or TM cells, suggesting a marked loss of translational regulation.
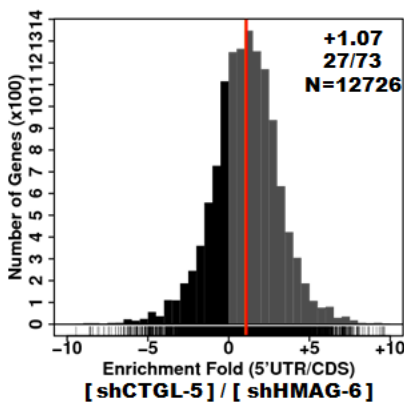

Fig. 2. *Comparing ribosome density in 5'UTR among two lines derived from the same parent cells and therefore with identical genetic background. Significant difference was observed in 5'UTR density, consistent with our result obtained from five other lines with different genetic background.*

We categorized genes that show significant density in the 5' UTR. Figure 3 shows such a list from a non-tumorigenic line. It is clear that these genes are distributed among a wide range of proteins performing distinct functions in the cell. We are working on completing this analysis for the other cells to compare differential regulation among these gene categories.

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Category	Term	Count
SP_PIR_KEYWORDS	phosphoprotein	489
SP_PIR_KEYWORDS	alternative splicing	425
UP_SEQ_FEATURE	splice variant	423
SP_PIR_KEYWORDS	nucleus	290
GOTERM_MF_FAT	GO:0046872~metal ion binding	237
SP_PIR_KEYWORDS	cytoplasm	219
SP_PIR_KEYWORDS	acetylation	200

Fig. 3. *Gene categories that show significant ribosome density in 5' UTR.* Hits in 5'UTRs of >1,400 different gene transcripts are present in this non-tumorigenic line.

Rosner lab: performed cell growth and characterizatios.
Pan lab: performed ribosome profiling and bioinformatics analysis.

Task 2 – Validate results for selected genes identified in task 1. → **In progress.**

As described under task 1b, we are in the process of completing the analysis of 5'UTR-derived translational regulation across non-tumorignic and tumorigenic cells. Our focus will be on these genes that are known to be involved in breast cancer development and proliferation.

We will validate changes in translational mis-regulation of several breast cancer relevant genes using reporter constructs.

Pan lab: will perform luciferase reporter assays during the period of no-cost extension (September 2012 – August 2013).

These results should enable validation of several potential gene regulation targets related to tumorigenic states. These results will also be needed to complete manuscript (i) outlined under Reported Outcomes.

Task 3 – Examine tumor initiating cell properties upon tRNA over-expression.
a) Test whether tRNA over-expression promotes cell proliferation and self-renewal in breast cells. → **Completed successfully when the last annual report was written in September 2011.**

Rosner lab: performed construction of tRNA over-expression cell lines and their characterizations.

Pan lab: performed tRNA abundance measurements.

We are currently writing manuscript (ii) outlined under Reported Outcomes. We plan to submit this manuscript within one month.

b) Test whether tRNA over-expression is associated with BT-IC differentiation. → **Unable to complete due to unexpected difficulties maintaining tRNA overexpression.**

Unexpectedly, we run into significant technical difficulties to maintain tRNA overexpression in our studies. We tried three approaches to over-express initiator-tRNA: transient transfection with tRNA transcripts (time required: one week), transient transfection with tRNA vector (time required: one week), and stable transfection with tRNA vector (time required: two months). Only stable transfection led to appreciable overexpression which enabled the characterization described under Task 3a. This result makes sense: tRNAs are derived from multiple gene copies and are already expressed at very high levels, so that increasing the existing amount in a cell even by ~1.5-fold requires a very high and sustained level of expression. For example, the initiator-tRNA has ten copies in a human genome and a breast cancer cell has at least one million initiator-tRNA molecules to begin with. The ~1.5-2 fold overexpression we were able to accomplish

requires an additional expression of over 500,000 molecules per cell, several orders of magnitude higher than what is typically needed for endogenous messenger RNA expression.

To our surprise and dismay, these cells lost tRNA overexpression over a period of a few weeks. We repeatedly attempted to re-establish cell lines that overexpress initiator-tRNA (we tried a total of four times, requiring ~ two months each time), and the same thing happened: initiator-tRNA overexpression was lost rapidly within a few weeks. At this time, we do not understand the reason for this inability of maintaining overexpression of initiator-tRNA. Using our initial tRNA-transfected cells, we did a number of assays including soft agar growth and mammosphere assays which were consistent with the idea that overexpression of tRNA promotes anchorage-independent cell growth and tumor initiation. Therefore we did not attempt to proceed further with this task which required a much more elaborate treatment course of mammosphere cultured cells.

Rosner lab: performed construction of tRNA over-expression cell lines and their characterizations.

Pan lab: performed tRNA abundance measurements.

Task 4 – Examine the effect of tRNA over-expression on tumor formation and metastasis in mice.
→ **Unable to complete due to unexpected difficulties maintaining tRNA overexpression.**
a) Test whether tRNA over-expression promotes invasion.
b) Determine the effect of tRNA over-expression on human BT-IC tumor formation and lung metastasis in mice.

Please see the description under Task 3b.

KEY RESEARCH ACCOMPLISHMENTS

→ Established a ribosome profiling method to look at active translation in human cells (year 1).
→ Demonstrated that over-expression of initiator-tRNA leads to increased cell proliferation and self-renewal in a breast epithelial cell line (year 1).
→ Determined that cells at distinct tumorgenic states have significantly different translational regulation through 5' UTR (year 2).

REPORTABLE OUTCOMES
→ Two manuscripts in preparation:
(i) Ribosome profiling analysis of translational regulation as a function of tumorigenic states (tasks 1 and 2);
(ii) Characterization of initiator-tRNA overexpression in breast cells (tasks 1 and 3a).

CONCLUSION
Our results show that tRNA over-expression results in increased cell proliferation and greater self-renewal potential in human breast cells. We have adapted a ribosome profiling method and have applied it to a panel of breast cancer and non-cancer breast cell lines. We have already determined that translational regulation through 5' UTR is significantly different depending on the

tumorigenic state of breast cells. We will complete the ribosome profiling analysis to identify genes whose translation is altered in breast cancer cells.

Chemotherapy, surgery, and radiotherapy are the current methods of choice to treat breast cancer. The effectiveness of these approaches is unquestionable, but they severely impact the physical and emotional health of the patient. Tremendous efforts are underway to precisely diagnose breast cancer subtypes and predict survival outcomes. This would allow the application of the most effective treatments possible while avoiding unnecessary therapies. We are working on identifying protein or RNA targets that are mis-regulated due to the high levels of tRNA found in breast cancer cells. These targets could serve as unique biomarkers in breast cancer. We also assess the physiological effects of tRNA over-expression on the development and progression of breast cancer. Our effort could establish the potential of tRNA and its regulatory targets as a new class of therapeutic targets.

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